INFECTION AND IMMUNITY, July 2005, p. 4127–4137 Vol. 73, No. 7
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.7.4127–4137.2005

Regulation of a Type III and a Putative Secretion System in *Edwardsiella tarda* by EsrC Is under the Control of a Two-Component System, EsrA-EsrB

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Received 12 November 2004/Returned for modification 22 January 2005/Accepted 7 March 2005

Edwardsiella tarda **is a gram-negative enteric pathogen that causes hemorrhagic septicemia in fish and gastro- and extraintestinal infections in humans. A type III secretion system (TTSS) and a putative secretion system (EVP) have been found to play important roles in *E. tarda* pathogenesis. Our previous studies suggested that the TTSS and EVP gene clusters were regulated by a two-component system of EsrA-EsrB. In the present study, we characterized another regulator, EsrC, which showed significant sequence similarity to the AraC family of transcriptional regulators. Mutants with in-frame deletions of *esrC* increased the 50% lethal doses in blue gourami fish, reduced extracellular protein production, and failed to aggregate. Complementation of *esrC* restored these three phenotypes. Two-dimensional gel electrophoresis showed that EsrC regulated the expression of secreted proteins encoded by the TTSS (such as EseB and EseD) and EVP (EvpC) gene clusters. The expression of *esrC* required a functional two-component system of EsrA-EsrB. EsrC in turn regulated the expression of selected genes encoded in TTSS (such as the transcriptional unit of *orf29*and *orf30*, but not *esaC*) and genes encoded in the EVP gene cluster. The present study sheds light on the regulation of these two key virulence-associated secretion systems and provides greater insight into the pathogenic mechanisms of this bacterium.**

Edwardsiella tarda is an important cause of hemorrhagic septicemia. It infects many commercially important farmed fish species and has led to extensive losses in both freshwater and marine aquaculture (43). The organism has a broad host range and is also known to cause infections in higher animals, including humans (33). In humans, it causes gastro- and extraintestinal infections such as myonecrosis (37), bacteremia (49), septic arthritis (31), and wound infections (1). The pathogenesis of *E. tarda* is multifactorial. Several potential virulence factors have been reported, including the abilities to invade epithelial cells (22, 26), resist serum (22, 26) and phagocyte-mediated killings (38), and produce toxins and enzymes such as hemolysins (18), catalases (40), and dermatotoxins (45) for disseminating infection.

Using a combination of comparative proteomics of secreted proteins and Tn*phoA* mutagenesis, we identified a type III secretion system (TTSS) (39, 42) and a putative secretion system (EVP) (for *E. tarda* virulence proteins) (41) that contributed to *E. tarda* PPD130/91 pathogenesis. TTSSs are generally used by bacterial pathogens to deliver virulence factors into host cells to subvert normal cell functions (21). The TTSS gene cluster of *E. tarda* PPD130/91 contains at least 30 open reading frames (ORFs) and is in two DNA regions (Y. P. Tan, J. Zheng, S. L. Tung, I. Rosenshine, and K. Y. Leung, unpublished data). The gene members of *E. tarda* TTSS are composed of apparatus, chaperones, effectors, and regulators. Some of the gene members of the *E. tarda* TTSS are homologous to other TTSSs encoded by pathogens such as enteropathogenic *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Yersinia enterocolitica*. The designation of the *E. tarda* TTSS genes is based on the sequence homologs in *Salmonella* pathogenicity island 2 (SPI-2) (16). The inactivation of the TTSS in *E. tarda* led to the increase of 50% lethal doses (LD_{50}) in blue gourami fish (39, 41).

The other gene cluster (EVP) is not unique to *E. tarda* but is widely distributed in many other animal and plant pathogens and symbionts such as *Salmonella*, *Vibrio*, *Yersinia*, *Escherichia*, *Rhizobium*, and *Agrobacterium* species with putative secretion functions (41). In *R. leguminosarum*, the impairment of EVP-like cluster affected the secretion of at least one protein based on two-dimensional gel electrophoresis (2-DE) analysis (2). Some proteins encoded in this EVP-like locus of *Rhizobium* shared homology with proteins encoded by the type III or type IV secretion systems (2). Folkesson et al. (11) also analyzed EVP homolog in *S. enterica* serovar Typhimurium and found that this gene cluster encoded putative cytoplasmic, periplasmic, and outer membrane localizing proteins. In *E. tarda*, eight genes (*evpA* to *-H*) have been found in the EVP cluster (41). *evpA* and *evpC* are under the control of a TTSS regulator *esrB*, while the secretion of EvpC is dependent on EvpB instead of on a functional TTSS. The deletion of *evpB* or *evpC* led to lower replication rates in gourami phagocytes and reduced protein secretion and virulence in blue gourami fish (41). Complementation of *evpB* and *evpC* deletion mutants restored the secretion of EvpC, partially increased the replication rates in gourami phogocytes and lowered the LD_{50} values in gourami fish, indicating that these *evp* genes are vital for *E. tarda* pathogenesis (41).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
E. tarda		
PPD130/91	Wild type; Km^s Col^r Amp^s $LD_{50} = 10^{5.0}$	38
Δ*esrC*	PPD130/91; in-frame deletion of *esrC* (missing amino acids 12 to 220)	This study
Δ*esrCN1*	PPD130/91; in-frame deletion of *esrC* (missing amino acids 2 to 72)	This study
Δ*esrCN2*	PPD130/91; in-frame deletion of *esrC* (missing amino acids 2 to 124)	This study
Δ*esrC163*	PPD130/91; in-frame deletion of *esrC* (missing amino acids 139 to 159)	This study
Δ*esrC263*	PPD130/91; in-frame deletion of *esrC* (missing amino acids 190 to 213)	This study
esrA	*esrA*::pFS-*esrA* (Km^r)	Unpublished data
esrB	*esrB*::Tn*phoA* (Km^r); $LD_{50} > 10^{8.0}$	39
pstC	*pstC*::Tn*phoA* (Km^r); $LD_{50} > 10^{8.0}$	39
306	Tn*phoA* mutant 306; Km^r; $LD_{50} > 10^{8.0}$	Unpublished data
Δ*esrC*+*esrC*	Δ*esrC* with pACYC + *esrC*	This study
E. coli		
JM109	Km^s Col^s Cm^s	Promega
MC1061 (λ*pir*)	*thi thr-1 leu-6 proA2 his-4 argE2 lacY1 galK2 ara-14 xy15 supE44 pir*	35
SM10 (λ*pir*)	*thi thr leu tonA lacY supE recA-RP4-2-Tc-Mu* Km^r *pir*	35
Plasmids		
pGEMT-Easy	Amp^r	Promega
pRE112	pGP704 suicide plasmid; *pir* dependent; Cm^r; *oriT oriV sacB*	9
pRE*ΔesrC*	pRE112 with *esrC* fragment deleted 12 to 220 amino acids	This study
pRE*ΔesrCN1*	pRE112 with *esrC* fragment deleted 2 to 72 amino acids	This study
pRE*ΔesrCN2*	pRE112 with *esrC* fragment deleted 2 to 124 amino acids	This study
pRE*ΔesrC163*	pRE112 with *esrC* fragment deleted 139 to 159 amino acids	This study
pRE*ΔesrC263*	pRE112 with *esrC* fragment deleted 190 to 213 amino acids	This study
pACYC184	Tet^r Cm^r	Amersham
pACYC+*esrC*	pACYC184 with wild type *esrC* gene	This study
pRW50	*lacZ* reporter vector; Tet^r	27
pRW*esrA*	pRW50 containing −711 to +89 of *esrA* relative to putative translational start site	This study
pRW*esrB*	pRW50 containing −879 to +89 of *esrB* relative to putative translational start site	This study
pRW*esrC*	pRW50 containing −911 to +89 of *esrC* relative to putative translational start site	This study
pRW*esaC*	pRW50 containing −876 to +110 of *esaC* relative to putative translational start site	This study
pRW*orf29*	pRW50 containing −667 to +101 of *orf29* relative to putative translational start site	This study
pRW*evpA*	pRW50 containing −711 to +89 of *evpA* relative to putative translational start site	This study
pRW*evpC*	pRW50 containing −822 to +92 of *evpC* relative to putative translational start site	This study
pRW*evpD*	pRW50 containing −562 to +92 of *evpD* relative to putative translational start site	This study
pRW*evpH*	pRW50 containing −746 to +89 of *evpH* relative to putative translational start site	This study
pQE60*lacI*	Derivative of pQE60; IPTG inducible; Amp^r	44
pQE*esrB*	pQE60*lacI* with wild-type *esrB* gene	This study
pQE*esrC*	pQE60*lacI* with wild-type *esrC* gene	This study

[a] Km, Kanamycin; Col, colistin; Amp, ampicillin; Cm, chloramphenicol; Tet, tetracycline. Superscripts: r, resistance; s, sensitivity.

The expressions of type III secretion apparatus and effectors are usually subjected to complicated regulation (12). Our previous studies suggested that EsrB regulated both the TTSS (such as *eseB* and *eseD*) and the EVP gene cluster (such as *evpA* and *evpC*) (41). In addition, the regulation of these two gene clusters is controlled by other factors such as temperature and the *pstSCAB-phoU* operon (41). This *pstSCAB-phoU* operon is a high-affinity phosphate-specific transport (PST) operon belonging to the family of ATP binding cassette (ABC) transporters (46). Our previous studies demonstrated that Tn*phoA* insertions in *pstB*, *pstC*, and *pstS* abolished the expression of TTSS, as well as EVP proteins, and the resulting PST mutants were highly attenuated in blue gourami fish (41). However, it is not clear how the two-component system and these factors regulate the TTSS and EVP locus.

A common mechanism of gene regulation in bacteria is via regulatory proteins of the AraC family. To date, this family contains more than 100 members as identified by sequence homology to the AraC protein of *Escherichia coli* (15). With a few exceptions, AraC homologs are transcriptional activators. We describe here the identification of EsrC, an AraC homolog encoded inside the TTSS. The markerless in-frame deletion mutation of *esrC* disrupted the expressions of the secreted proteins of the TTSS and EVP locus. The phenotype of the Δ*esrC* mutant is similar to the phenotypes of the *esrA* and *esrB* mutants. Our studies showed that EsrA-EsrB regulates the expression of the secreted proteins of TTSS and EVP proteins through *esrC*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in the present study are shown in Table 1. *E. tarda* stains were grown in tryptic soy broth (TSB; Difco/Becton Dickinson) or Dulbecco modified Eagle medium (DMEM) at 25°C without shaking while *Escherichia coli* strains were cultured in Luria broth (LB; Difco/Becton Dickinson) at 37°C. Cultivation of bacteria in DMEM was carried out at 5% (vol/vol) CO_2 atmosphere. When required, the appropri-

TABLE 2. Oligonucleotides used in this study

Name	Sequence[a] (5′ to 3′)
MutC-for	AT**GGTACC**CTGTCCAGGCTGA TCA ATGCC
MutC-int-rev	GAAGGCTGGCCCGACGACCGGTGACGAGCAGTAGATGCAA
MutC-rev	AT**GGTACC**AACCCGCGAC CTTGGCGGTCA
MutC-int-for	CGTCGGGCCAGCCTTCACCAC
MutC-N-for	AT**GGTACC**TACGCTGCGCCAAGCGCTG
N-rev	ATGGCGATGGGGAGCGGCATGAGGTGCTCCTGACTGAGGT
MutC-N-rev	AT**GGTACC**AGAATGGCTTCGCAGCGGGTAAC
N-for	CCGCTCCCCATCGCCAT
N2-rev	GTGTCGAACAGGTCGGCCATGAGGTGCTCCTGACTGAGGT
N2-for	GCCGACCTGTTCGACACGCT
MutC-HTH-for	AT**GAGCTC**TCGACGCCGAGGATCTCCTG
HTH-rev	ATGCCGAACTTGTCGTTGGGTTCGAGCCGATGGCGGTAGA
MutC-HTH-rev	AG**GAGCTC**ACGGCCAGCTCATTCAGGGTGC
HTH-for	AACGACAAGTTCGGCATGG
MutC-HTH2-for	AT**GGTACC**GGGTCGACTGCGCCTTATT
HTH2-rev	TTCGCTCGGTGACTGACTCTTTTTAGAGGTGGTTTCGAGT
MutC-HTH2-rev	AT**GGTACC**GTTGTAGATCAGCAGGGCC
HTH2-for	AGTCAGTCACCGAGCGAAG
EsrB-com-for	AT**GAATTC**ATTAAAGAGGAGAAATTAACCATGACTATTTCTATTTTGCCTCTG
EsrB-com-rev	AT**GGATCC**TATTAAAACTCCAGAACCCCCAGG
EsrC-com-for	AT**GAATTC**ATTAAAGAGGAGAAATTAACCATGCCCAATTTGCATCTACTGCTC
EsrC-com-rev	AT**GGATCC**TATTAGCCGGCGCGGTGGTGAAGGCTG

[a] Restriction enzyme sites used for cloning of PCR products are labeled in boldface; the overlapping sequences or ribosome-binding sites included in primers are underlined.

ate antibiotics were supplemented at the following concentrations: ampicillin (100 μg/ml); kanamycin (50 μg/ml); colistin (12.5 μg/ml); chloramphenicol (30 μg/ml); and tetracycline (10 μg/ml).

Construction of deletion mutants and plasmids. Overlap extension PCR (19) was used to generate in-frame deletion of *esrC* on the *E. tarda* PPD130/91 chromosome. For the construction of Δ*esrC*, two PCR fragments were generated from PPD130/91 genomic DNA with the primer pairs of MutC-for plus MutC-int-rev, and MutC-rev plus Mut-int-for (Table 2). The resulting products generated a 754-bp fragment containing the upstream of *esrC* and a 751-bp fragment containing the downstream of the *esrC*, respectively. A 16-bp overlap in the sequences (underlined) permitted amplification of a 1,505-bp product during a second PCR with the primers MutC-for and MutC-rev, both of which were introduced into a KpnI restriction site (boldface text in Table 2), respectively. The resulting PCR product contained a deletion from nucleotides 34 to 660 of *esrC*. The PCR product was cloned into pGEMT-Easy vector, and DNA sequencing was performed to confirm that the construct was correct. The Δ*esrC* fragment was excised with KpnI, ligated into suicide vector pRE112 (Cm^r) (9), and the resulting plasmid then transformed into SM10λ *pir*. The single-crossover mutants were obtained by conjugal transfer into *E. tarda* PPD130/91. Double-crossover mutants were obtained by plating onto 10% sucrose–tryptic soy agar plates. The deletion mutants were confirmed by PCR and sequencing. The construction of other *esrC* deletion mutants followed a similar protocol. The primers used for the construction of Δ*esrCN1* are MutC-N-for plus N-rev and MutC-N-rev plus N-for. The primers used for the construction of Δ*esrCN2* are MutC-N-for plus N2-rev and MutC-N-rev plus N2-for. The primers used for Δ*esrC163* are MutC-HTH-for plus HTH-rev and MutC-HTH-rev plus HTH-for. The primers used for Δ*esrC263* are MutC-HTH2-for plus HTH2-rev and MutC-HTH2-rev plus HTH2-for. The corresponding restriction sites (KpnI or SacI) are labeled in boldface in Table 2.

For the construction of pACYC+*esrC*, the complete *esrC* gene was obtained by PCR with *Pfu* Turbo polymerase (Stratagene) with *E. tarda* PPD130/91 as the template DNA. The PCR products were digested and ligated into ScaI- and EcoRI-digested pACYC184 plasmid. For the construction of pQE*esrB* and pQE*esrC*, the complete *esrB* and *esrC* genes were amplified by using the forward primer *esrB*-com-for or *esrC*-com-for containing the ribosome-binding site (underlined) and EcoRI site (boldface) and the reverse primer *esrB*-com-rev or *esrC*-com-rev which supplied the BamHI site (boldface) (Table 2). The PCR products were digested with BamHI and EcoRI and cloned into EcoRI- and BamHI-digested PQE60*lacI* (44).

For the construction of LacZ reporter plasmids, the putative promoter regions were amplified from *E. tarda* PPD130/91 genomic DNA and the PCR products were cloned into pGEMT-Easy vector. The resulting plasmids were sequenced, and the inserts were cut with BamHI and EcoRI (or MfeI) and subcloned into BamHI- and EcoRI-digested pRW50 plasmid (27).

LD_{50} determinations. Healthy naive blue gourami (*Trichogaster trichopterus* Pallas) of ca. 14 g were obtained from a commercial fish farm and infected with *E. tarda* as described previously (26). The mortality of the fish was recorded over a period of 7 days after infection. The LD_{50} values were calculated by the method of Reed and Muench (34).

Phagocyte isolation. Phagocytes were isolated from the head kidney of naive gourami and purified according to the procedure of Secombs (36) and as described by Srinivasa Rao et al. (38). Purified phagocytic cells (4×10^6 to 5×10^6 cells/well) were allowed to adhere to 48-well tissue culture plates (Falcon) in an L-15 medium (Sigma) that was supplemented with 5% fetal bovine serum. After 3 h of incubation at 25°C in a 5% (vol/vol) CO_2 atmosphere, the cells were washed twice with Hanks balanced salt solution (HBSS; Sigma) and infected with *E. tarda* at a multiplicity of infection of 1:1 and incubated at 25°C for 30 min. The cells then were washed twice with HBSS and incubated with 100 μg of gentamicin/ml to kill all of the extracellular bacteria for 90 min. The monolayers were then washed twice with HBSS and lysed with 1% Triton X-100, and viable bacterial counts were enumerated by plate count. Phagocyte replication ratios were calculated by dividing the viable bacterial count after 5 h by the 2-h bacterial count.

2-DE and protein identification. Protein isolation and 2-DE were performed as previously described (41). Protein spots were identified with matrix-assisted laser desorption ionization–time of flight as described earlier (25).

β-Galactosidase assays. Bacterial were grown in DMEM (for *E. tarda*) or LB (for *E. coli*) overnight at 25°C or 37°C. β-Galactosidase activities were determined with cells permeabilized with sodium dodecyl sulfate and chloroform as described by Miller (29).

RESULTS

Sequence analysis of regulators. The TTSS of *E. tarda* is composed of two regions and was subjected to the regulation of a two-component regulator EsrA-EsrB encoded within region 2 (Fig. 1A) (Tan et al., unpublished). The deduced amino acid sequence of EsrA has a high homology to a number of two-component system sensors, including SpiR (SsrA) of *Salmonella enterica* serovar Typhimurium (31% identity and 45% similarity to residues 29 to 834 of EsrA), which controls the

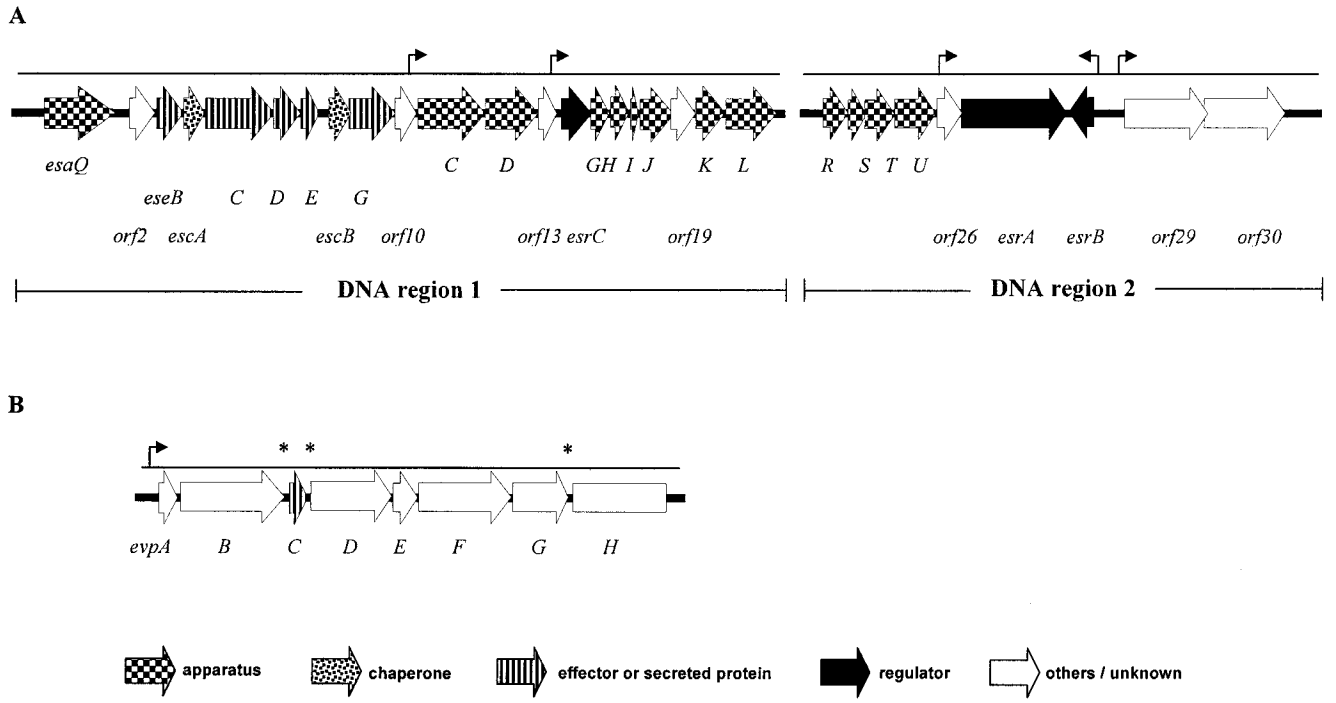

FIG. 1. Schematic presentation of TTSS and EVP gene clusters of *E. tarda* PPD130/91. (A) TTSS gene cluster; (B) EVP gene cluster. The arrows indicate confirmed promoters, and the asterisks denote the corresponding *lacZ* fusions constructed, but no promoter activities were detected.

TTSS of SPI-2 (30), and MmoS of *Methylococcus capsulatus* (31% identity and 48% similarity to residues 411 to 905 of EsrA), which is involved in the copper-dependent regulation of a soluble methane monooxygenase (5). The response regulator EsrB has a CheY-like receiver domain at the N-terminal half and a helix-turn-helix (HTH) domain at the C-terminal half. The homologs of EsrB include the response regulator SsrB of the two-component system SsrA-SsrB of *S. enterica* serovar Typhimurium (42% identity and 57% similarity to residues 25 to 214 of EsrB) (3) and the response regulator YvqC of the two-component system YvqE-YvqC of *Bacillus subtilis* (30% identity and 50% similarity to residues 8 to 208 of EsrB) (47).

The characterization of the regulation of the two TTSS regions of *E. tarda* is ongoing, and we have found that *esrC* encoded another regulator. *esrC* is oriented in the same transcriptional direction as *esaG*, *esaH*, *esaI*, *esaJ*, *orf19*, *esaK*, and *esaL* (Fig. 1A) and are presumably in the same transcriptional unit since no obvious transcription termination sequences have been found between them. The putative ribosome-binding site (AGGAGC) was identified 6 bp upstream of the postulated translational initiation codon of *esrC*. A homology search of EsrC retrieved a large number of bacterial transcriptional regulators of the AraC family. Some of them are regulators of TTSSs, including ExsA of *Pseudomonas aeruginosa* (30% identity and 59% similarity to residues 133 to 229 of EsrC) (13) and VirF of *Y. enterocolitica* (32% identity and 63% similarity to residues 136 to 221 of EsrC) (4) (Fig. 2). Members of this protein family, as exemplified by AraC, contain the dimerization and effector binding domains in the N-terminal half and two potential HTH DNA-binding and transcriptional activation domains at the C-terminal half. The similarity among these proteins is higher at the C-terminal half, which is supposed to be a DNA-binding domain (Fig. 2). However, no homology was found within the 126 N-terminal amino acids of EsrC in a search of the databases.

Role of EsrC in *E. tarda* virulence and protein secretion. The homology of EsrC to the TTSS transcriptional activators suggests that EsrC may be required for the virulence of *E. tarda*. A deletion mutant, Δ*esrC*, was constructed to remove an internal fragment of 627 bp, and the resulting mutant contained the first 33 bp and the last 30 bp of the *esrC* gene. The Δ*esrC* mutant increased its LD_{50} (2.6 logs higher than that of the wild type) in blue gourami fish and showed a lower replication rate in blue gourami phagocytes (Table 3). Δ*esrC* was also found to secrete less than 10% of the extracellular proteins (ECPs) and did not show any aggregation in DMEM after 24 h of culturing (Table 3). In general, the phenotypes of the Δ*esrC* mutant are similar to those of *esrA* and *esrB* insertional mutants (Table 3). Complementation of the Δ*esrC* deletion mutant in *trans* with a plasmid-borne wild-type copy of *esrC* (Δ*esrC*+*esrC*) restored the wild-type phenotypes, including lower LD_{50} values, an increase in the replication rate inside the blue gourami phagocytes, autoaggregation, and the production of normal amounts of ECPs (Table 3).

To show the importance of the N-terminal sequence and the two HTHs on the function of EsrC, four additional in-frame deletion mutants were constructed: Δ*esrCN1* (deletion of 71 amino acids from the N-terminal end), Δ*esrCN2* (deletion of 123 amino acids from the N-terminal end), Δ*esrC163* (deletion of the first HTH domain), and Δ*esrC263* (deletion of the second HTH domain) (Table 1). All of these mutants significantly reduced the LD_{50} values, and the values were comparable to

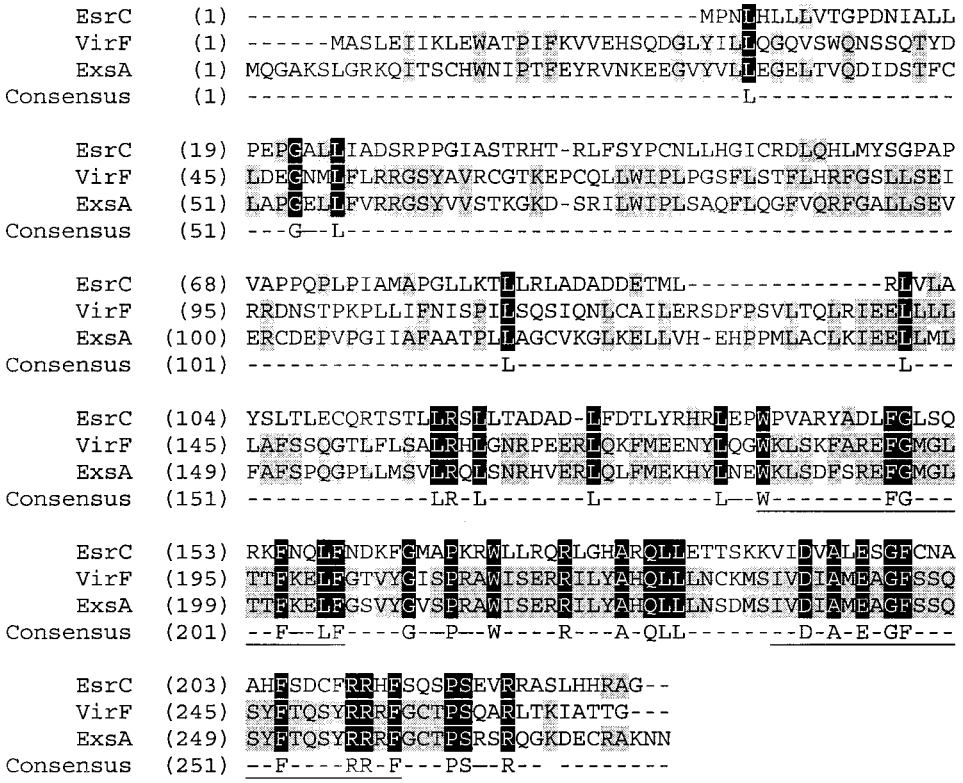

FIG. 2. Amino acid sequence alignments of the EsrC and other members of the AraC family of transcription regulatory proteins of TTSSs, including VirF (*Y. enterocolitica*) and ExsA (*P. aeruginosa*). Black boxes denote identical residues, and shaded boxes indicate conserved substitutions. The underlining indicates the putative HTH domains.

those of the Δ*esrC* deletion mutant (Table 3). All four of these Δ*esrC* mutants also had low ECP production and failed to aggregate in DMEM culture (Table 3). Our results demonstrate that EsrC plays an important role in *E. tarda* pathogenesis and that an intact and complete protein is required for it to function.

To find out which genes are regulated by EsrC, the total bacterial proteins and the ECPs of the wild type and the Δ*esrC* mutant were compared by using 2-DE (Fig. 3A and B). Four protein spots (spots 1 to 4) were absent in the Δ*esrC* mutant. Spots 1 and 2 were confirmed with matrix-assisted laser desorption ionization–time of flight mass spectrometry as the TTSS secreted proteins EseB and EseD, respectively. Spots 3 and 4 were identified as EvpA and EvpC of the EVP proteins, respectively. The two secreted proteins EseC (spot 5) and E2 (spot 6) reported previously (41) were also absent in the ECP profile of the Δ*esrC* mutant (Fig. 3C and D).

TABLE 3. Characterization of mutants derived from *E. tarda* PPD130/91

Strain	Mean ECP concn (μg/ml) ± SD	Aggregation[a]	Macrophage replication[b]	LD_{50}
PPD130/91	4.50 ± 0.23	Y	++	$10^{5.0}$
esrA	0.25 ± 0.08	N[d]	+[d]	$10^{7.6}$[d]
esrB	0.53 ± 0.08[e]	N[e]	ND[c]	$10^{8.0}$[e]
Δ*esrC*	0.19 ± 0.05	N	+	$10^{7.6}$
Δ*esrCN1*	0.18 ± 0.07	N	ND	$10^{7.8}$
Δ*esrCN2*	0.21 ± 0.08	N	ND	$10^{7.5}$
Δ*esrC163*	0.17 ± 0.08	N	ND	$10^{7.6}$
Δ*esrC263*	0.19 ± 0.06	N	ND	$10^{7.6}$
Δ*esrC*+*esrC*	4.30 ± 018	Y	++	$10^{5.4}$

[a] For the aggregation phenotype in DMEM, Y denotes aggregation and N denotes no aggregation.
[b] For the macrophage replication assay, ++ denotes bacterial normal replication and + denotes bacterial reduced replication.
[c] ND, not determined.
[d] Tan et al., unpublished.
[e] Srinivasa Rao et al. (41).

Functional relationship between two-component system EsrA-EsrB and EsrC. The observation that *esrA*, *esrB*, and *esrC* mutants had similar low ECP productions (which were <10% of the wild-type strain) in DMEM (Table 3) led us to examine their interactions in regulating the TTSS of *E. tarda*. EsrA-EsrB is a putative two-component system that regulates the expressions of the TTSS and EVP gene clusters. However, the opposite orientation of *esrA* and *esrB* in the chromosome makes their transcriptional relationship elusive. To investigate the relationships of *esrA*, *esrB*, and *esrC*, we examined the effect of loss-of-function mutations in each of them on the expression of *esrA*, *esrB*, and *esrC*. Plasmids pRW*esrA*, pRW*esrB*, and pRW*esrC* carrying the *lacZ* transcriptional fusions to the upstream (putative promoter) regions of *esrA*, *esrB*, and *esrC*, respectively, were constructed (Fig. 1A) and introduced into the wild type and the *esrA*, *esrB*, and Δ*esrC* mutants. High β-galactosidase activities were detected in all of the wild-type

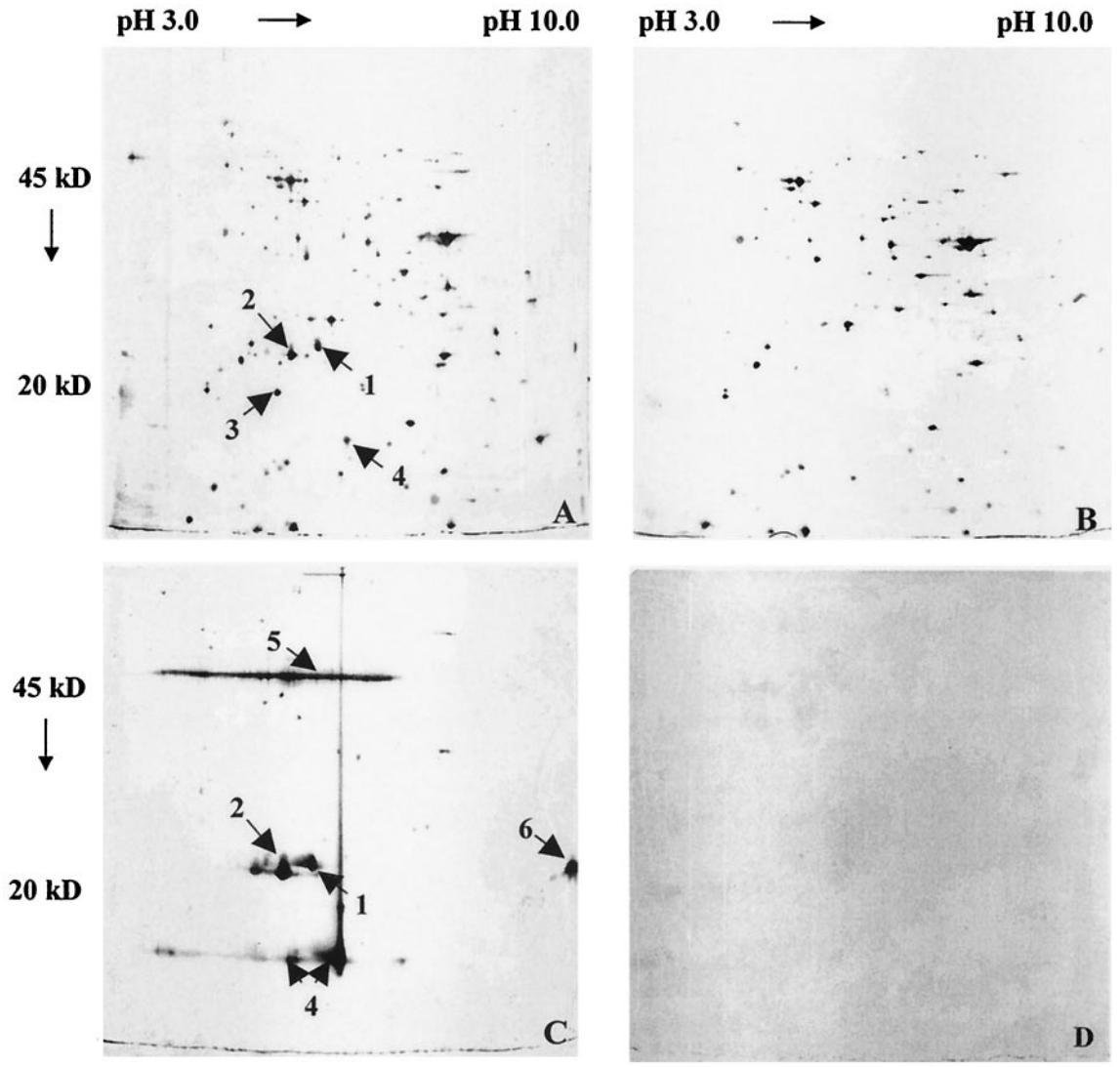

FIG. 3. Proteome analysis of *E. tarda* PPD130/91 and the Δ*esrC* mutant. Total protein (A and B) and ECP (C and D) fractions in DMEM were separated on Immobiline DryStrips (pH 3 to 10) combined with 2-DE analysis (12.5% polyacrylamide). Strains: A and C, wild type; B and D, Δ*esrC* mutant. Portions (30 μg) of samples were loaded for the total protein fractions. ECP fractions were adjusted according to the relative secreted protein amount of each strain as follows: wild type (20 μg) and Δ*esrC* mutant (2 μg). Gels were silver stained.

strains containing one of these pRW50 derivatives, indicating the presence of promoters in front of *esrA*, *esrB*, and *esrC* (Fig. 4). The inactivation of *esrA* did not reduce the β-galactosidase expression of *esrB-lacZ*, and the inactivation of *esrB* also did not decrease the expression of the *lacZ* of *esrA-lacZ* (Fig. 4). When the plasmid pRW*esrC* was introduced into *esrA* and *esrB* mutants, the expression of β-galactosidase was reduced ~100-fold compared to that of the wild type and was comparable to the vector control pRW50. Our results therefore suggest that EsrA and EsrB exert their regulatory effect upstream on *esrC*. The inactivation of *esrA*, *esrB*, and *esrC* in the chromosome did not affect the expression of *esrA-lacZ*, *esrB-lacZ*, and *esrC-lacZ*, respectively, indicating that they may not be self-regulated (Fig. 4). To determine whether EsrB directly activates *esrC* expression, the effect of providing *esrB in trans* under the control of *lacI* on the expression of *esrC-lacZ* from pRW*esrC* was tested in *E. coli*. Upon the induction of IPTG (isopropyl-β-D-thiogalactopyranoside), pQE*esrB* was able to activate *esrC-lacZ* expression in *E. coli*. Very low LacZ activity was detected without the IPTG induction (Table 4). Therefore, *esrB* may directly activate the expression of *esrC*.

Regulation of TTSS apparatus genes and *orf29* and *orf30*. To find out whether *esrC* regulates TTSS apparatus genes, we examined the expression of *esaC* in the Δ*esrC* mutant. EsaC is homologous to SsaC of *Salmonella* SPI-2, which is a TTSS apparatus protein. The inactivation of *esaC* abolished the secretion of EseB, EseC, and EseD (data not shown). Plasmid pRW*esaC* carrying the *lacZ* fusion with the promoter region of *esaC* (Fig. 1A) was introduced into the wild-type *E. tarda* and *esrA*, *esrB*, and Δ*esrC* mutants. High β-galactosidase activities were detected in the wild type, confirming the presence of a promoter. However, expression of the *lacZ* fusion was reduced

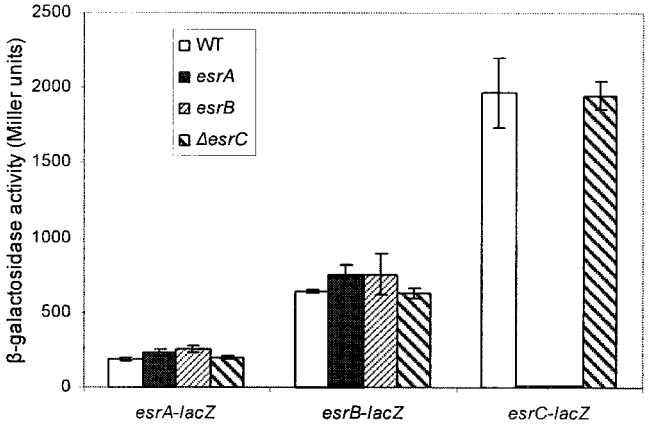

FIG. 4. Effect of loss-of-function mutants in regulatory proteins on the expression of *esrA*, *esrB*, and *esrC*. Levels of transcription of the different reporter gene fusions in different *E. tarda* genetic backgrounds were measured by assaying β-galactosidase activities in bacterial cell lysates. The values represent the means ± the standard deviations (SD) from one representative experiment performed with triplicate samples. Equivalent results were obtained at least in triplicates.

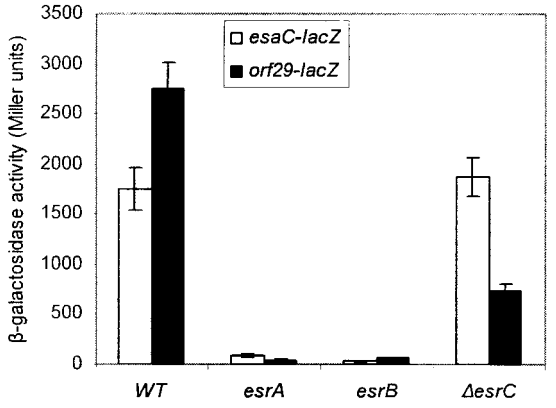

FIG. 5. Effect of loss-of-function mutants in regulatory proteins on the expression of *esaC* and *orf29*. Levels of transcription of the different reporter gene fusions in different *E. tarda* genetic backgrounds were determined by assaying β-galactosidase activities in bacterial cell lysates. The values represent the means ± the SD from one representative experiment performed with triplicate samples. Equivalent results were obtained at least in triplicates.

>20-fold in the *esrA*, and *esrB* mutants but not in the Δ*esrC* mutant (Fig. 5). Our results suggest that the EsrA-EsrB two-component system regulates the expression of *esaC* and that *esrC* is not required for *esaC* expression.

Plasmid pRW*orf29* carrying the *lacZ* fusion with the promoter region of *orf29* was also constructed (Fig. 1A) and introduced into *esrA*, *esrB*, and Δ*esrC* mutants. As shown in Fig. 5, expression of *lacZ* of *orf29-lacZ* in the Δ*esrC* mutant was found to be four times lower than that in the wild type, demonstrating regulation by EsrC on the transcriptional unit of *orf29* and *orf30*. Expression of *lacZ* of *orf29-lacZ* was also examined in the background of *esrA* or *esrB*, and the β-galactosidase activities in these two mutants were also reduced (Fig. 5), indicating that EsrA-EsrB also can regulate the transcriptional unit of *orf29* and *orf30* independent of EsrC. Therefore, it is likely that there are two promoters upstream of *orf29*: one is *esrC* dependent, and the other is EsrB dependent. Expressions of *orf29* and *orf30* were regulated by both EsrB and EsrC and could be activated from either of these promoters.

EsrC regulates the EVP gene cluster. EvpA and EvpC were shown to be regulated by EsrC earlier in the 2-DE studies (Fig. 3). The next question is how *esrC* regulates the expression of EVP genes and whether EsrC also regulates the other genes encoded in the EVP gene cluster. Plasmid pRW*evpA* containing the putative promoter region of *evpA* (Fig. 1B) was introduced into the wild-type *E. tarda* and the Δ*esrC* mutant. As shown in Fig. 6, high β-galactosidase activities were detected in the wild type, which confirmed the presence of a promoter. Expression of the LacZ reporter decreased over 30 times in the Δ*esrC* mutant compared to the wild type (Fig. 6). The data further confirmed the regulation of EsrC on the *evpA-lacZ* and indicated that the regulation was at the transcriptional level. To find out whether there were other possible promoters in this EVP gene cluster, we constructed three more reporter fusions in pRW50 plasmids with the upstream sequences of *evpC*, *evpD*, and *evpH* (Fig. 1B). These constructs were introduced into the *E. tarda* wild type, and the *lacZ* expression was monitored. The results showed that the expressions of reporters in all of the wild-type *E. tarda* with these three constructs had low β-galactosidase activities that were comparable to the

TABLE 4. Expression of reporter fusions with *esrB* or *esrC* in *E. coli*

Plasmid(s)	β-Galactosidase activity ± SD[a]	
	No IPTG	IPTG
pRW*esrC*	11 ± 2	13 ± 3
pQE*esrB* + pRW*esrC*	19 ± 3	4,475 ± 1,075
pRW*evpA*	24 ± 4	15 ± 3
pQE*esrC* + pRW*evpA*	11 ± 2	1,498 ± 36

[a] That is, the β-galactosidase activities (in Miller units) present in *E. coli* JM109 containing different plasmids.

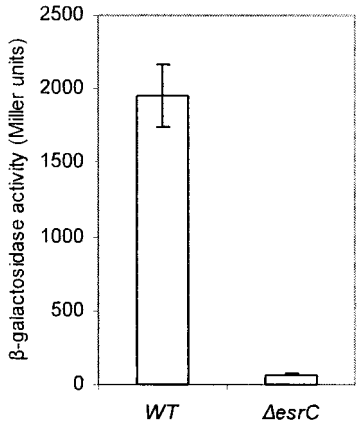

FIG. 6. Effect of Δ*esrC* on the expression of *evpA*. Levels of transcription of the reporter gene fusion in different *E. tarda* genetic backgrounds were measured by assaying β-galactosidase activities in bacterial cell lysates. The values represent the means ± the SD from one representative experiment performed with triplicate samples. Equivalent results were obtained at least in triplicates.

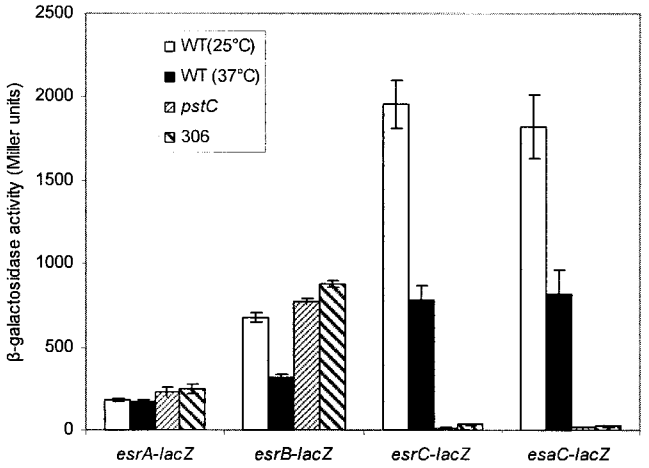

FIG. 7. Effect of temperature, *pstC*, and mutant 306 on the expression of *esrA*, *esrB*, *esrC*, and *esaC*. *E. tarda* strains with different reporter fusions were cultured in 25 or 37°C in DMEM. Levels of transcription of the different reporter gene fusions were measured by assaying β-galactosidase activities in bacterial cell lysates. The values represent the means ± the SD from one representative experiment performed with triplicate samples. Equivalent results were obtained at least in triplicates.

vector control (pRW50) (data not shown). The results indicate that there may not be other promoters in the regions examined. It is possible that the EVP gene cluster transcript in one single unit and *esrC* is required for the expression of all of the genes in this EVP gene cluster. To determine whether EsrC directly activates *evpA* expression, the effect of providing *esrC* in *trans* under the control of *lacI* on the expression of *evpA-lacZ* was tested in *E. coli*. Under the induction of IPTG, pQE*esrC* was able to activate *evpA-lacZ* in *E. coli*, while very low LacZ activity was detected without the IPTG induction (Table 4). Therefore, *esrC* may directly activate the expression of *evpA*.

Regulation of *esrA-esrB* and *esrC*. The expression of TTSS secreted proteins and EVP proteins was temperature dependent (41), and EsrC was speculated to contribute to the receiving of temperature change signals due to the similarity to thermo-induced regulator VirF in yersiniae (Fig. 2). However, there is no evidence to support this hypothesis. To clarify this, plasmids pRW*esaC* and pRW*esrC* were transformed into the *E. tarda* wild type, and the expression level of *lacZ* was studied at either 37 or 25°C. As shown in Fig. 7, the expression of *lacZ* of *esrC-lacZ* and *esaC-lacZ* at 37°C decreased approximately two times compared to that seen at 25°C. These data indicate that the expression of *esrC* itself is affected by temperature. Since we had shown earlier that *esrC* was under the control of *esrA* and *esrB*, we wanted to determine whether the effect of temperature on the *esrC* transcription was the result of the decrease in *esrA* and *esrB* expression. The expression of *lacZ* in the wild type of *E. tarda* carrying pRW*esrA* and pRW*esrB* at 37 and 25°C was examined. We found that the expression of *lacZ* of the *E. tarda* wild type carrying *esrA-lacZ* was similar at either 37 or 25°C. However, the expression of *lacZ* by the wild type carrying *esrB-lacZ* was two times lower at 37°C than that at 25°C, demonstrating that the expression of *esrB*, but not *esrA*, was regulated by temperature. These results suggest that the effect of temperature on the expressions of *eseB*, *eseD*, and *evp* genes is through regulating the expression of *esrB*.

The insertion of Tn*phoA* in *pst* genes has been reported to affect the expression of TTSS and EVP genes (41). On the other hand, a Tn*phoA* mutant (i.e., mutant 306, accession no. AY643479) was found to be highly attenuated when injected into blue gourami fish, and it failed to produce TTSS (EseB and EseD) and EVP proteins (EvpA and EvpC) (data not shown). Sequencing data at the point of transposon insertion of this mutant showed similarity to a putative membrane protein of *Y. pestis*. Therefore, we were very interested in exploring how these mutants affected this regulatory hierarchy. To this end, the plasmids of pRW*esrA*, pRW*esrB* and pRW*esrC* were transformed into *pstC* and mutant 306. As shown in Fig. 7, in both *pstC* and mutant 306, the expressions of *lacZ* of *esrA-lacZ* and *esrB-lacZ* did not decrease although *eseB*, *eseD*, *evpA*, and *evpC* failed to express in these mutants (data not shown) (41). In sharp contrast, the expression of *esrC-lacZ* was suppressed significantly. These results indicate that the effect of *pstC* and mutant 306 on the expression of TTSS and *evp* genes might not be through regulating the transcription of *esrA* and *esrB*. We further examined the expression of the TTSS apparatus gene *esaC* in *pstC* and mutant 306. Interestingly, in both of these mutants, the expression of *lacZ* of *esaC-lacZ* decreased significantly. Such observation indicates that the insertion of Tn*phoA* in *pstC* and the putative membrane protein also affects TTSS apparatus gene expression.

DISCUSSION

EsrC is a positive regulator. We report here the characterization of another novel regulatory protein, EsrC, which is encoded within the TTSS of *E. tarda*. EsrC has a significant sequence similarity to members of the AraC family of the transcriptional regulatory proteins. A nonpolar deletion mutation in *esrC* rendered the mutant less virulent. The wild-type phenotypes could be restored by the complementation of *esrC* provided by a plasmid with a constitutive promoter. Our results suggest that *esrC* is an essential virulence gene for *E. tarda* pathogenesis, and its role is comparable to *esrA* and *esrB* (Table 3). The LacZ reporter fusion analysis indicated that EsrC was downstream of the EsrA-EsrB regulatory pathway (Fig. 8). The regulation of EsrB on *esrC* was possibly direct since EsrB could regulate the expression of the reporter fusion (*esrC-lacZ*) in *E. coli* (Table 4). *esrC* was possibly in the same transcriptional unit and under the same mode of regulation with the apparatus genes (Fig. 8). *esrC* was also shown to be not self-regulated (Fig. 4). The TTSS apparatus genes, therefore, may be regulated by EsrA-EsrB instead of EsrC. This hypothesis was further confirmed by the reporter *esaC-lacZ*, which was expressed in the Δ*esrC* mutant but not in the *esrA* or *esrB* insertional mutants (Fig. 5). Although both EsrC and the two-component system EsrA-EsrB control the TTSS, EsrA-EsrB may directly regulate the expression of TTSS apparatus genes, whereas it is EsrC that regulates the expression of some specific transcriptional units such as the secreted proteins of TTSS (e.g., EseB and EseD). The regulation of EsrA-EsrB on TTSSs is, therefore, different from SsrA-SsrB in *Salmonella* species, in which SsrA-SsrB controls the expression of genes encoding both secreted proteins and the TTSS apparatus. The difference

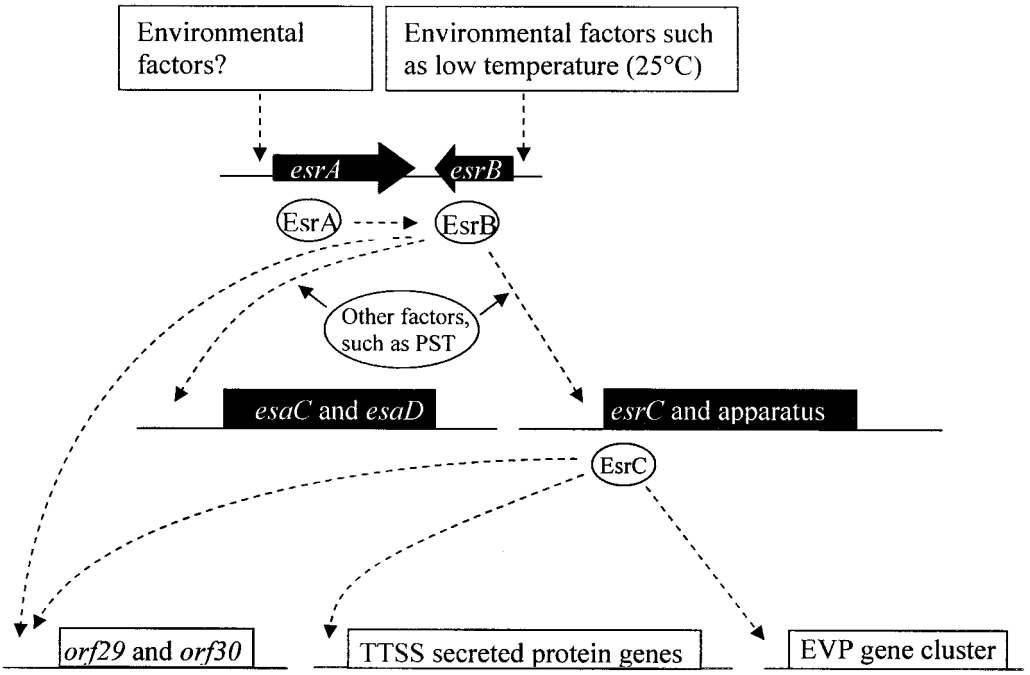

FIG. 8. Model for the regulation of TTSS and EVP gene clusters by EsrA, EsrB, and EsrC in *E. tarda* PPD130/91. Growth in DMEM at 25°C favors the expression of *esrA* and *esrB*. EsrA is speculated to phosphorylate EsrB, and the accumulation of activated EsrB leads to the expressions of *esrC* and downstream TTSS apparatus genes, as well as *orf29* and *orf30*. EsrC then activates the expressions of TTSS secreted proteins, *orf29* and *orf30*, and the EVP gene cluster. The regulation of EsrB on the TTSS apparatus genes or *esrC* transcription was subjected to the modulation of other factors such as the PST operon. Other possible unknown environmental factors are indicated in the diagram.

may be due to the lack of another regulatory protein encoded within TTSS of SPI-2.

EsrC regulates *orf29* and *orf30* and EVP. The expression of *orf29* and *orf30*, the two novel genes identified at the end of the TTSS, was controlled by both EsrA-EsrB and EsrC, which might indicate that they were part of the TTSS gene cluster (Fig. 1A and 8). The regulation of these two ORFs is different from that of other TTSS apparatus genes (e.g., *esaC*), which are directly regulated only by the EsrA-EsrB two-component system. *orf29* and *orf30* may have a special function for TTSSs. The analysis with COILs (http://www.ch.embnet.org/software/coils_form.html) showed that the predicted proteins had a high degree of coils. Pallen et al. (32) reported that many of the secreted proteins of the TTSS shared a common coiled-coil structure feature. *orf29* and *orf30* share no homology with known structure proteins of the TTSS that are believed to be conserved in different bacterial species. Therefore, it is likely that *orf29* and *orf30* do not encode for apparatus but instead are effector proteins of *E. tarda*. This possibility will be examined in a future study.

The EVP gene cluster is encoded outside of the TTSS and mutation in *esrB* has been reported to affect the expression of EvpA and EvpC (41). However, the secretion of EVP proteins is independent of the TTSS. Mutations in the putative translocon proteins of the TTSS did not affect the secretion of EvpC (41). The EsrA-EsrB two-component system regulates EVP via EsrC (Fig. 8). The LacZ reporter study suggests that EsrC regulated the transcription of the *evpA-H* transcriptional unit; only one promoter sequence was found (Fig. 1 and 6), and the regulation of EsrC on *evpA* is possibly direct (Table 4). The homologs of the EVP gene cluster are widely distributed in other pathogens such as *Escherichia*, *Salmonella*, *Vibrio*, and *Yersinia* species with unknown functions (41), and it is possible that these homologs may also be regulated by the regulators encoded in the respective TTSSs. The direct regulation of the EVP gene cluster by the regulators encoded within the TTSS of *E. tarda* suggests the functional relationship between the TTSS and the EVP gene cluster. The EVP gene cluster and its homologs encode several conserved core proteins and possibly encode a novel secretion system (2, 11, 41). This is the first report to suggest a communication between the TTSS and the EVP in *E. tarda*. In *Salmonella* species, SPI-5 encodes at least five effectors (48), among which SigD/SopB is coordinately regulated with SPI-1 genes (6) and is secreted by the SPI-1 TTSS (20). However, PipB, another effector in SPI-5, is part of the SPI-2 regulon and is translocated by the SPI-2 TTSS to the *Salmonella*-containing vacuole (23). These studies demonstrate a functional and regulatory cross talk between different secretion systems. *E. tarda* might use EsrC to coordinate the EVP secretion system with the TTSS in infection or survival in host cells.

Involvements of other regulators. The regulators encoded within the TTSS usually respond to regulation by environmental factors or other global regulators. In *Salmonella* species, the SsrA-SsrB two-component system was regulated by another two-component system OmpR-EnvZ, of which the response regulator OmpR binds directly to the *ssrA* and *ssrB* promoter (10, 24). In *E. tarda*, the transposon mutant 306, which has an insertion in a putative membrane protein, was also shown to affect the expression of the TTSS. Unlike OmpR-EnvZ in

Salmonella species, this mutant did not affect the expressions of *esrA* or *esrB* but suppressed the expression of *esrC* and the TTSS apparatus genes (e.g., *esaC*) (Fig. 7). Interestingly, transposon insertion in *pstC* also has a similar effect on the expressions of TTSS and EVP (Fig. 7), suggesting that the PST operon has some control over the expressions of TTSS and EVP. The PST operon is required for phosphate transport (46). In *S. enterica* serovar Typhimurium, the transposon mutant *pstS* reduced the expression of the TTSS regulator of *hilA* and the invasion genes, and this repression was due to the negative control of the PhoR-PhoB two-component system. The *pstS* mutation led to the accumulation of PhoB~P, and PhoB~P directly or indirectly repressed *hilA* and the invasion genes (28). *E. tarda* might have a similar mechanism: the insertion inactivation of the PST system may lead to the accumulation of some negative regulators of the TTSS that have not been identified, thus repressing the expression of TTSS genes. If this is the case, this negative regulation would not modulate the expressions of *esrA* and *esrB* since both of their expressions were not affected in the *pstC* mutant background (Fig. 7). This negative regulator might directly interact with EsrB and interrupt its function. Another hypothesis is that some proteins encoded in the PST operon function as positive regulators and coordinate with EsrB in regulating the TTSS. However, we have shown that in *E. coli*, under the induction of IPTG, pQE*esrB* could activate *lacZ* expression of *esrC-lacZ* because of large amounts of EsrB accumulation. In *E. tarda*, as a regulator gene, the expression of *esrB* was low and EsrB could not activate the expression of the TTSS without the help of the positive regulator encoded in the PST operon. Future studies will attempt to distinguish between these possibilities.

Effect of high temperature. The expression of the *esrB-lacZ* fusion decreased substantially at 37°C compared to that seen at 25°C, but there was no change for the *esrA-lacZ* fusion at different temperatures. Our results suggest that the regulation of temperature on the TTSS is through the modulation of *esrB* expressions (Fig. 8). It is possible that the primary regulatory event is at the *esrB* transcription and that the accumulation of EsrB increases the transcription of *esrC*. This results in the increased production of EsrC activator proteins that bind to sites upstream of the target genes and activate transcription. If so, the mechanism response for temperature sensing is independent of EsrB and EsrC. EsrA functions as a sensor in EsrA-EsrB two-component system, and it is possible that EsrA is the one that responds to temperature changes. However, the expression of *esrB-lacZ* in an *ersA* mutant background did not decrease compared to that in the wild-type background at 25°C (Fig. 4). From these data, we hypothesize that there must be proteins other than EsrA responding to temperature changes. Alternatively, temperature changes may affect DNA conformation in the *esrB* promoter region. Changes in DNA topology have been shown to affect expressions of a variety of genes (7, 8, 14, 17). In *E. tarda*, the topology of the DNA promoter region of *esrB* may vary at different temperatures and result in altered *esrB* expressions. Further experiments must be done to confirm these two hypotheses.

In conclusion, we report that the EsrA-EsrB/EsrC regulatory cascade is the key regulon controlling the expressions of TTSS and EVP gene clusters and plays a vital role in *E. tarda* pathogenesis. EsrA-EsrB controls most of the transcriptional units of the TTSS to ensure that the TTSS is functional. EsrA-EsrB also controls EsrC, which regulates a subset of transcriptional units. They may be effector proteins or proteins for special functions, such as the transcriptional units of *orf29* and *orf30*. Another main role of EsrC is to control the EVP, possibly another secretion system that is different from the TTSS. The EsrA-EsrB/EsrC regulatory cascade, therefore, can coordinate the expressions of these two secretion systems during infection and host-bacterium interactions. The sequencing of the TTSS and EVP gene clusters is ongoing. More transcriptional units will be identified, and this will help to dissect the precise roles of this EsrA-EsrB/EsrC regulatory cascade. Future work will also be carried out to identify and characterize the factors affected by the insertional disruptions of *pstC* and the putative membrane protein and to identify factors that communicate temperature changes with the TTSS and EVP gene clusters. This information will be useful in elucidating the functions of TTSS and EVP gene clusters and thus the pathogenesis of *E. tarda*.

ACKNOWLEDGMENTS

We are grateful to the Biomedical Research Council of Singapore (BMRC), A*STAR, the National University of Singapore, and the Lee Hiok Kwee Donation for providing research grants in support of this study.

We thank S. J. Busby of University of Birmingham for providing the plasmid pRW50 and its sequence information. We also thank I. Rosenshine and P. Tang for critical comments, which helped in improving the manuscript.

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Editor: J. B. Bliska